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PACAP protects neuronal differentiated PC12 cells against the neurotoxicity induced by a mitochondrial complex I inhibitor, rotenone

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Abstract **In vivo and in vitro studies have suggested a neuroprotective role for Pituitary adenylate cyclase activating polypeptide (PACAP) against neuronal insults. Here, we showed that PACAP27 protects against neurotoxicity induced by rotenone, a mitochondrial complex I inhibitor that has been implicated in the pathogenesis of Parkinson's disease (PD). The neuroprotective effect of PACAP27 was dose-dependent and blocked by its specific receptor antagonist, PACAP6-27. The effects of PACAP27 on rotenone-induced cell death were mimicked by dibutyryl-cAMP (db-cAMP), forskolin and prevented by the PKA inhibitor H89, the ERK inhibitor PD98059 and the p38 inhibitor SB203580. PACAP27 administration blocked rotenone-induced increases in the level of caspase-3-like activity, whereas could not restore mitochondrial activity damaged by rotenone. Thus, our results demonstrate that PACAP27 has a neuroprotective role against rotenone-induced neurotoxicity in neuronal differentiated PC12 cells and the neuroprotective effects of PACAP are associated with activation of MAP kinase pathways by PKA and with inhibition of caspase-3 activity; the signaling mechanism appears to be mediated through mitochondrial-independent pathways.**

Keywords: Pituitary adenylate cyclase polypeptide; Rotenone; Pheochromocytoma cells; Parkinson's disease

1. Introduction

Parkinson's disease (PD) is a motor disorder characterized by bradykinesia, akinesia, rigidity and tremors [1,2]. Idiopathic PD results from the degeneration of dopaminergic neurons in the substantia nigra. Inhibition of mitochondrial complex I activity has been implicated in the pathogenesis of PD [3] and has been used extensively as a model system with which to elicit neurochemical alterations associated with PD. Inhibition of complex I activity results in ATP inhibition and loss of mitochondrial membrane potential, leading to the death of nigral neurons [4]. Rotenone, a mitochondrial complex I inhibitor, can recapitulate the behavioral [5] and neuropathological hallmarks of PD [6,7], including induction of apoptosis and acceleration of α-synuclein formation in PD models in vivo [8] and in vitro [9]. Hence, these features of rotenone make it a useful model of PD both in vivo and in vitro.

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first isolated from hypothalamic extracts on the basis of its ability to stimulate adenylate cyclase activity in pituitary cells. PACAP has been designated to the VIP/secretin/glucagon family of peptides because of its 68% identity with vasoactive intestinal peptide [10,11]. There are two forms of mammalian PACAP (27 or 38 amino acid residues) and both have been shown to have similar biological activity and receptor-binding activity. PACAP receptors belong to the family of G protein-coupled receptors with seven transmembrane domains, and there are at least three types of PACAP receptors: PAC1, VPAC1 and VPAC2. The PAC1 receptor is mainly localized in the central nervous system and the anterior pituitary, while VPAC1 and VPAC2 receptors are concentrated in peripheral tissues, such as the lung, kidney and liver. The PAC1 receptor is coupled to adenylate cyclase (AC) and phospholipase C, through AC activation and it elevates cAMP and activates protein kinase A (PKA), which can activate the mitogen-activated protein kinase (MAPK) pathway. However, PACAP can also activate the MAPK pathway independently of AC activation [12,13].

Recent studies have indicated that PACAP is a promising neuroprotective peptide and that it may play an important role during nervous system development and during regeneration following nervous system injuries [14]. PACAP has also been shown to protect against apoptosis induced by neurotoxins including glutamate [15,16], beta-amyloid [17], 6-hydroxydopamine (6-OHDA) [18] and human prion protein [19]. Finally, in vitro studies have shown that PACAP can stimulate neurite outgrowth [20].

In the present study, we investigated the effects of PACAP27 on rotenone-induced neurotoxicity using neuron-like PC12 cells in vitro in which the expression of PACAP-specific PAC1 receptor was confirmed [17,21]. We investigated the influence of PACAP27 on caspase-3 activity (which plays a crucial role in apoptosis of PC12 cells) and whether PACAP27 could restore mitochondrial integrity after damage by rotenone. We further investigated whether the protective effects

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Abbreviations: db-cAMP, dibutyryl-cAMP; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; H89, *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; MAP, mitogen-activated protein; Myr-ΨPKC, myristoyl-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp-Ile; PACAP, pituitary adenylate cyclase activating polypeptide; PKA, protein kinase A; PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate

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of PACAP27 are sensitive to drugs affecting PKA, extracellular signal-regulated kinase (ERK) and p38 MAPK signaling.

2. Materials and methods

2.1. Chemicals

Rotenone, PACAP27, PACAP6-27, MTT, DMSO, dibutyryl-cAMP (db-cAMP), forskolin, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), H-89 and myristoyl-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp-Ile (Myr-Ψ) protein kinase C (PKC) were purchased from Sigma (St. Louis, MO, USA); PD98059, SP600125 and SB203580 were purchased from Biomol (Plymouth Meeting, PA, USA); Annexin-V and propidium iodide (PI) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Apo-ONE™. Homogeneous Caspase-3/7 Assay kits were purchased from Promega (Madison, WI, USA); JC-1 and Hoechst 33258 were purchased from Molecular Probes (Eugene, OR, USA).

2.2. Cell culture

Rat pheochromocytoma (PC12) cells were obtained from the cell bank of Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 5% (v/v) horse serum (Gibco BRL, Grand Island, NY, USA) and 5% (v/v) newborn calf serum (Gibco BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin in a 75 ml vented culture flask (Corning, Acton, MA). The cultures were maintained in 5% CO_2/95% humidified air at 37 °C. Medium was changed every 2 days. By 3–4 days of incubation in the flask, cells had reached 70–80% confluence and were seeded onto a 10 mm-diameter 96-well plate at a cell density of 1×10^4/well for test.

2.3. Cell survival

PC12 cells were treated and cultured for 24 h in the absence or presence of rotenone (50–5000 nM), PACAP27 (10^{-13}–10^{-6} M), PACAP6-27 (10^{-5} M), db-cAMP (a cAMP analog) (10^{-4} M), forskolin (10^{-6} M), TPA (a PKC activator) (10^{-7} M), H-89 (a PKA inhibitor) (10^{-6} M), Myr-ΨPKC (a PKC inhibitor) (10^{-6} M), PD98059 (an ERK inhibitor) (2×10^{-5} M), SP600125 (a JNK inhibitor) (5×10^{-5} M) or SB203580 (a p38 inhibitor) (4×10^{-5} M). After 24 h of drug treatment, cells were incubated for 4 h with 5 g/l MTT and then DMSO (200 μl) was added for 15 min. MTT reduction was quantified at 570 nm using a micro-plate reader (Bio-Tek Instruments Inc., Canada). For quantification of surviving cells, free-floating cells pooled with cells detached by mild trypsinization were incubated in 100 μl annexin-V-FLUOS buffer solution containing 2 μl Annexin-V and 2 μl PI reagent for 10–15 min at 20 °C and then cooled so that the reaction was terminated. FITC-fluorescence densities were measured with a FACS Calibur flow cytometer. Flow cytometry used an excitation wavelength of 488 nm. To detect morphological changes of PC12 cells after 24 h incubations, cultured cells were visualized on a fluorescence microscope. For evaluation of morphological changes to the nuclei (e.g., apoptosis), cells were stained with Hoechst 33258 and visualized by fluorescence microscopy.

2.4. Caspase-3-like activity

Caspase-3-like activity in culture was measured using an Apo-ONE™ Homogeneous Caspase-3/7 Assay Kit (Promega) according to the manufacturer's instructions. Briefly, cells (5×10^4 cells/well) in type I collagen-coated 96-well plates (Corning) were rinsed twice with phosphate-buffered saline (PBS). The cultures were incubated with or without the indicated stimulants in DMEM (50 μl) at 37 °C in an atmosphere of 95% air and 5% CO_2. Cells were lysed in 50 μl of Homogeneous Caspase-3/7 Buffer containing the caspase-3 substrate Z-DEVD-rhodamine 110, and the cell lysates were incubated for 4 h at room temperature. Fluorescence intensity (excitation at 498 nm and emission at 521 nm) was measured with an safire2 spectrofluorophotometer (Tecan Genios Plus, Switzerland).

2.5. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was quantified using the ratiometric probe JC-1 (Molecular Probes). In healthy PC12 cells, the intact membrane allows the lipophilic dye JC-1 to enter into the mitochondria where it accumulates and aggregates, producing an intense orange signal. In apoptotic cells, where the mitochondrial membrane potential has collapsed, the monomeric JC-1 remains in the cytosol and appears green. PC12 cells were treated for 24 h with rotenone (250 nM) in the absence or presence of PACAP. Cells were incubated for 30 min with 10 μg/ml JC-1 at 37 °C under 5% CO_2, and then washed twice with 0.1 M PBS. The proportion of aggregated vs. monomeric JC-1 probe was quantified using the ratio of fluorescence emissions at 590 nm (orange) over 530 nm (green) with a FACS Calibur flow cytometer.

2.6. Data analysis

Statistical analyses were done by SPSS software using a one-way ANOVA, followed by a two-tailed Student's *t*-test or multiple comparison test where appropriate. A $P < 0.05$ was considered significant for all analyses.

3. Results

3.1. PAC1 receptors mediate effects of PACAP27 on rotenone-induced cytotoxicity

Chronic exposure of PC12 cells to rotenone resulted in a concentration-dependent decrease of cell viability as assayed by MTT at 24 h, indicating a reduction in the mitochondria of living cells (Fig. 1G). Observed morphological changes associated with increasing rotenone exposure, included cell shrinkage, disappearance of neurites, nuclear condensation and DNA fragmentation (Fig. 1A–F). PACAP27 is believed to act as a potent neurotrophic factor in the nervous system. When co-treated with rotenone, a significant ($P < 0.05$) increase of MTT was observed at concentrations of 10^{-12}–10^{-7} M (Fig. 1H). However, when PACAP27 was administered to PC12 cells with PACAP6-27 (10^{-5} M), a potent PAC1 receptor antagonist, in presence of rotenone, its protective effects largely disappeared (Fig. 1I). Taken together, these results indicate that the effects of PACAP27 are mediated through the activation of PAC1 receptors in PC12 cells. In fact, extremely low concentrations exerted marked effects on the biological activity of the cells. Additionally, because cytotoxicity induced by rotenone is mainly evidenced by apoptosis rather necrosis, FACS and Annexin-V-PI double staining were used to detect apoptotic cells. We found that PACAP not only decreased numbers of apoptotic cells, but promoted cells to transform from late apoptosis to early apoptosis (Fig. 2).

3.2. PKA and ERK/p38 MAPK transduction pathways involved in neuroprotective effects of PACAP27

To determine which signaling pathways are involved in the protective effects of PACAP27, we investigated whether the protective effects of PACAP27 were influenced by stimulators or inhibitors of different protein kinases. In this series of experiments, we found that db-cAMP (10^{-4} M), the cell permeant cAMP analog and forskolin (10^{-6} M), a potent PKA stimulator, markedly inhibited the effects of rotenone-induced cytotoxicity, as compared to TPA (10^{-7} M), a potent PKC stimulator (Fig. 3A). Co-administration of the selective PKA inhibitor *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide (H89) (10^{-6} M) with rotenone (250 nM) and PACAP27 (10^{-8} M) inhibited the effect of PACAP27. However, the selective PKC inhibitor, Myr-ΨPKC (10^{-6} M) had no effect (Fig. 3B).

Previous studies have revealed that MAPK is involved in PACAP-evoked neuroprotection against various neuronal insults. However, studies examining which MAPK subtype is

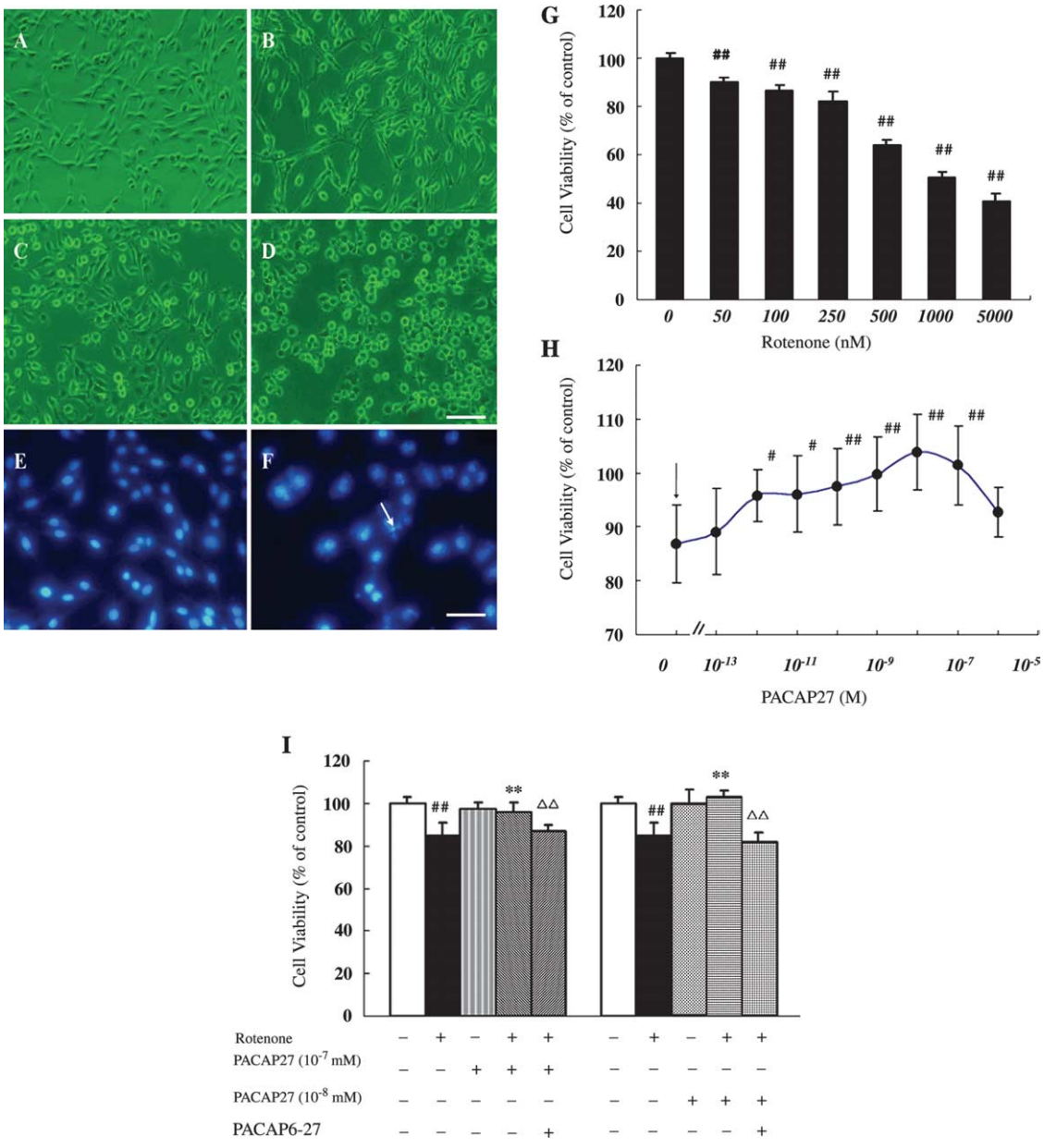


Fig. 1. Rotenone-induced cell death and neuroprotective effects of PACAP27 in PC12 cells. (A)–(D) Photomicrographs of PC12 cells after exposure to various concentrations of rotenone by fluorescence microscope without any stain and show dose-dependent effects of rotenone. (A) Control cells. (B) Cells treated with rotenone (50 nM) for 24 h. (C) Cells treated with rotenone (100 nM) for 24 h. (D) Cells treated with rotenone (250 nM) for 24 h. (E) and (F) Photomicrographs of PC12 cells stained with Hoechst 33258 and visualized by fluorescence microscopy. (E) Control cells. (F) Cells treated with rotenone (250 nM) for 24 h; (A)–(F) note the morphological changes associated with increasing rotenone exposure, including cell shrinkage, disappearance of neurites, nuclear condensation and DNA fragmentation (↓), Bar = 100 μm. (G) Dose-dependent neurotoxicity of rotenone from 0 to 5000 nM for 24 h by MTT detection. The data are represented as means ± S.D. of four experiments. $^{\#\#}P < 0.01$ vs. Control group. (H) Neuroprotective effects of PACAP27 against rotenone-induced neurotoxicity of PC12 cells. The data are represented as means ± S.D. of four experiments. $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ vs. rotenone (250 nM) group (↓). I: PACAP27 protects PC12 cells from rotenone-induced toxicity and PACAP6-27, an antagonist of PACAP receptor, blocks its protective effects. The data are represented as means ± S.D. of four experiments. $^{\#\#}P < 0.01$ vs. control group. $^{**}P < 0.01$ vs. rotenone (250 nM) group. $^{\triangle\triangle}P < 0.01$ vs. PACAP27 (10^{-8} M)-treated group with rotenone (250 nM).

involved are rarely reported. Thus, we designed PC12 cells exposed to ERK, JNK and p38 inhibitors with rotenone and PACAP27 and found that PD98059 (2×10^{-5} M), SB203580 (5×10^{-5} M), but not SP600125 (4×10^{-5} M), markedly inhibited PACAP27-induced neuroprotection (Fig. 3C). These results demonstrate that the neuroprotective effects of PACAP27 are mediated through PKA and ERK/p38, but not PKC or JNK, signaling pathways.

3.3. PACAP27 blocks rotenone-induced impairments on caspase-3-like activity and does not rescue mitochondrial membrane potential

Because the activation of caspase-3 occurs following various neuronal insults and under certain pathological conditions, we examined caspase-3-like activity in PC12 cells by measuring the ability of cell lysates to cleave a flourometric caspase-3 substrate, Z-DEVD-Rhodamine 110. After 24 h of exposure to

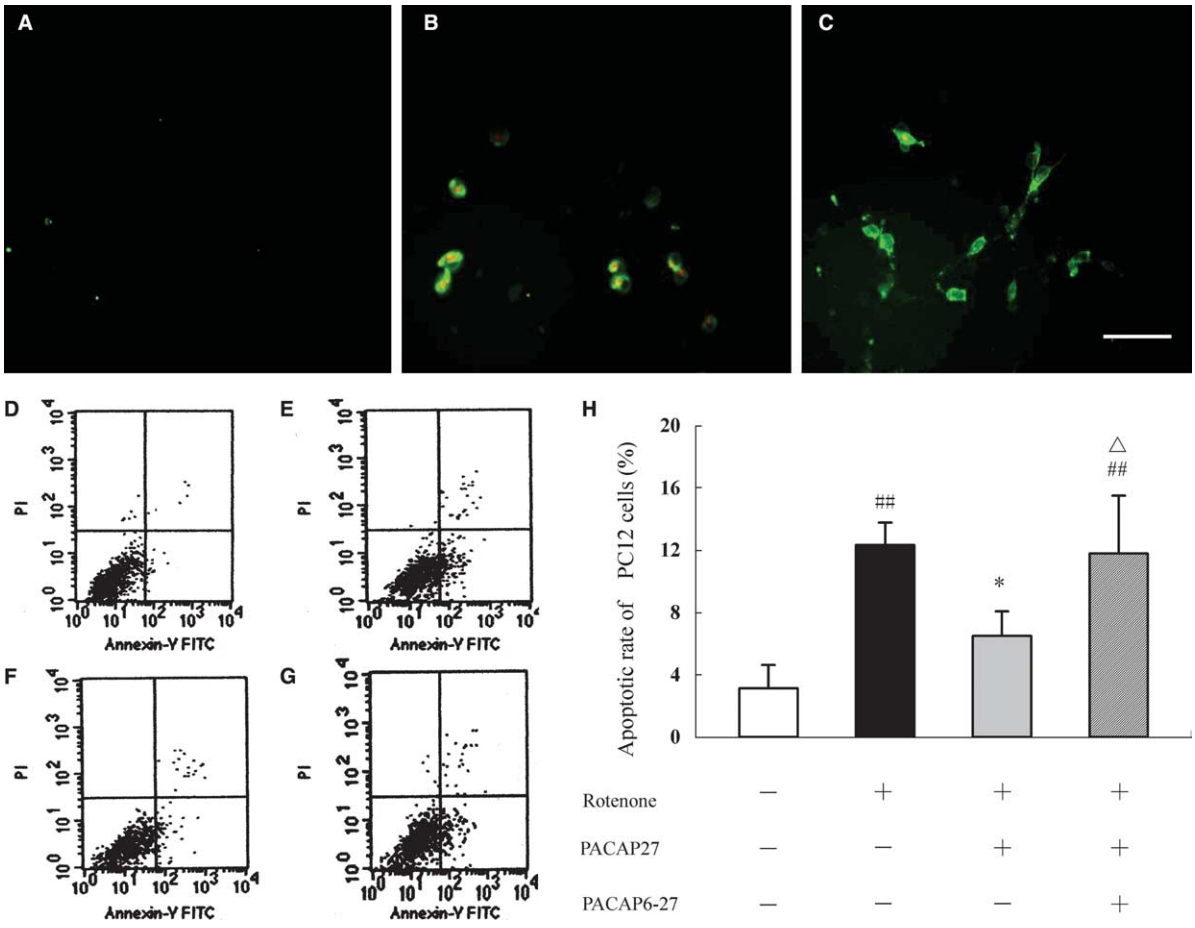


Fig. 2. PACAP27 markedly inhibits rotenone-induced apoptosis in PC12 cells. (A)–(C) Micrographs of PC12 cells with fluorescent images with Annexin V-PI double-staining. (A) Control cells. (B) Cells treated with rotenone (250 nM) for 24 h. (C) Cells treated with rotenone (250 nM) and PACAP27 (10^{-8} M) for 24 h. Bar = 100 μm. (D)–(G) Micrographs of PC12 cells by flow cytometry. (D) Control. (E) Cells treated with rotenone (250 nM) for 24 h. (F) Cells treated with rotenone (250 nM) and PACAP27 (10^{-8} M). (G) Cells treated with rotenone (250 nM) and PACAP27 (10^{-8} M) in the presence of PACAP6-27 (10^{-5} M). (H) Apoptotic rate of PC12 cells (%) by flow cytometry FACS. Each value represents the means ± S.D. of three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control. $^{*}P < 0.05$ vs. rotenone (250 nM) group. $^{\triangle}P < 0.05$ vs. PACAP27 (10^{-8} M)-treated group with rotenone (250 nM).

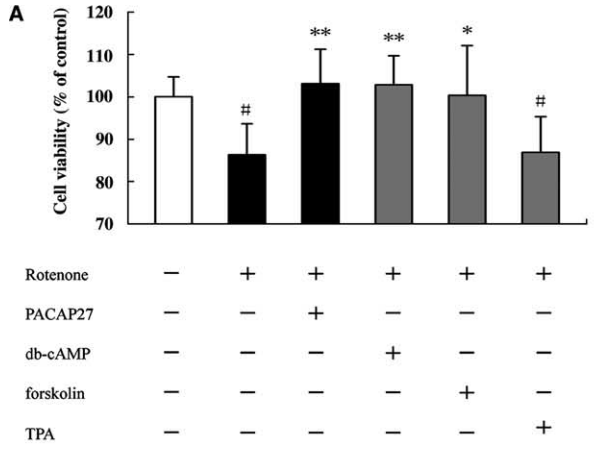
rotenone (250 nM), the level of caspase-3-like activity was markedly increased compared to control groups. Exposure of these cells to PACAP27(10^{-8}–10^{-7} M) reduced the basal level of caspase-3-like activity and provoked a significant ($P < 0.05$) reduction of rotenone-evoked caspase-3-like activation. This effect was reversed by PACAP6-27 (10^{-5} M). Thus, the present results suggest that PACAP27-induced neuroprotection against rotenone-induced cytotoxicity may be mediated via caspase-3 deactivation (Fig. 4).

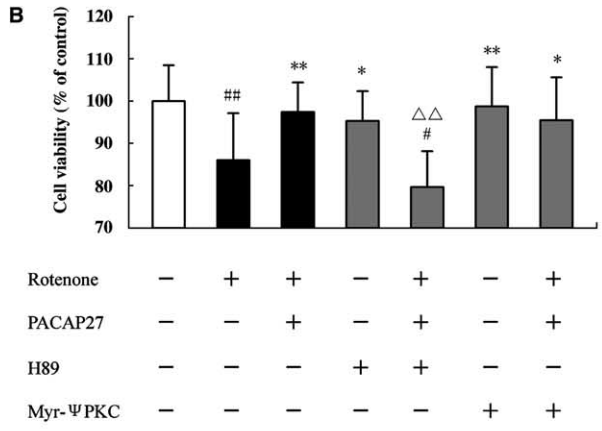
In order to determine whether rotenone-induced apoptosis of PC12 cells could be ascribed to an alteration of mitochondrial activity and the relationship between caspase-3 deactivation and mitochondrial damage, the membrane potential of mitochondria was measured using the ratiometric probe JC-1. Rotenone treatment for 24 h caused a marked decrease of mitochondrial membrane potential. However, addition of PACAP27 (10^{-7}–10^{-8} M) did not rescue the membrane potential, nor did it restore mitochondrial activity (Fig. 5). Taken together, the data indicate that inhibition of caspase-3 activity by PACAP27 is likely mediated through mitochondrial-independent pathways.

4. Discussion

In this study, we determined that PACAP27 increased cell survival against the neurotoxic effects of rotenone, a mitochondrial toxin implicated in the pathogenesis of PD. We demonstrated that PACAP protects differentiated PC12 cells against rotenone-induced apoptosis and that the neuroprotective effect of PACAP is associated with: (1) activation of MAPK by PKA; and (2) inhibition of caspase-3 activity mediated through a mitochondrial-independent pathway.

The neuroprotective effects of PACAP have been demonstrated on various neurotoxic agents which induce apoptosis and/or necrosis, such as glutamate, ethanol, ceramide, beta-amyloid and 6-OHDA. Our study indicates that the mitochondrial complex I inhibitor, rotenone, provoked the death of PC12 cells in a concentration-dependent manner. Rotenone-induced cell death had the characteristic features of apoptosis including cell shrinkage, nuclear condensation and DNA fragmentation, which is consistent with a recent study by Liu et al. [22]. The neuroprotection of PACAP was effective at extremely low concentrations (10^{-12} M), suggesting that PACAP27 could act as a





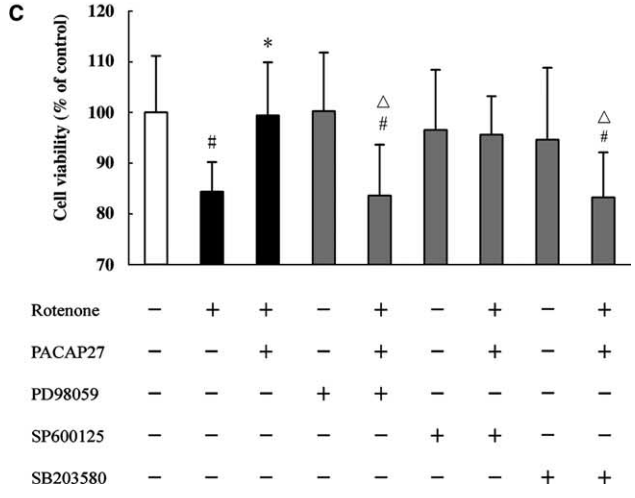


Fig. 3. Involvement of protein kinase cascades in the neuroprotective effects of PACAP27. (A) Effects of PKA/PKC activators on rotenone-induced cell death in PC12 cells. The cells were cultured for 24 h with rotenone in the presence or absence of db-cAMP (10^{-4} M)/forskolin (10^{-6} M) or TPA (10^{-7} M). (B) Effects of the selective PKA/PKC inhibitors on PACAP27-evoked neuroprotection. PC12 cells were treated with rotenone and PACAP in the absence or presence of the protein kinase inhibitor (10^{-6} M) for 24 h. (C) Effects of selective MAPK inhibitors on PACAP27-mediated neuroprotection. PC12 cells were treated with rotenone and PACAP in the absence or presence of each protein kinase inhibitor for 24 h; cell viability was assessed by MTT. The data are represented as means ± S.D. of four experiments. $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ vs. the control group. $^{**}P < 0.01$ and $^{*}P < 0.05$ vs. rotenone-treated group. $^{\triangle\triangle}P < 0.01$ and $^{\triangle}P < 0.05$ vs. PACAP27-treated group with rotenone (250 nM).

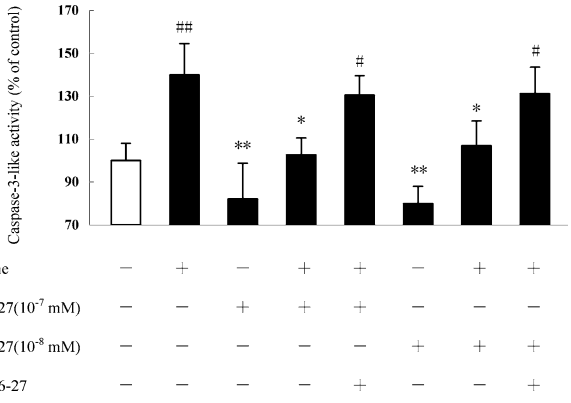


Fig. 4. Effects of PACAP27 on rotenone-evoked caspase-3 activity. $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ with respect to the control group. $^{**}P < 0.01$ and $^{*}P < 0.05$ with respect to rotenone (250 nM)-treated group. Data are presented as means ± S.D. of three experiments.

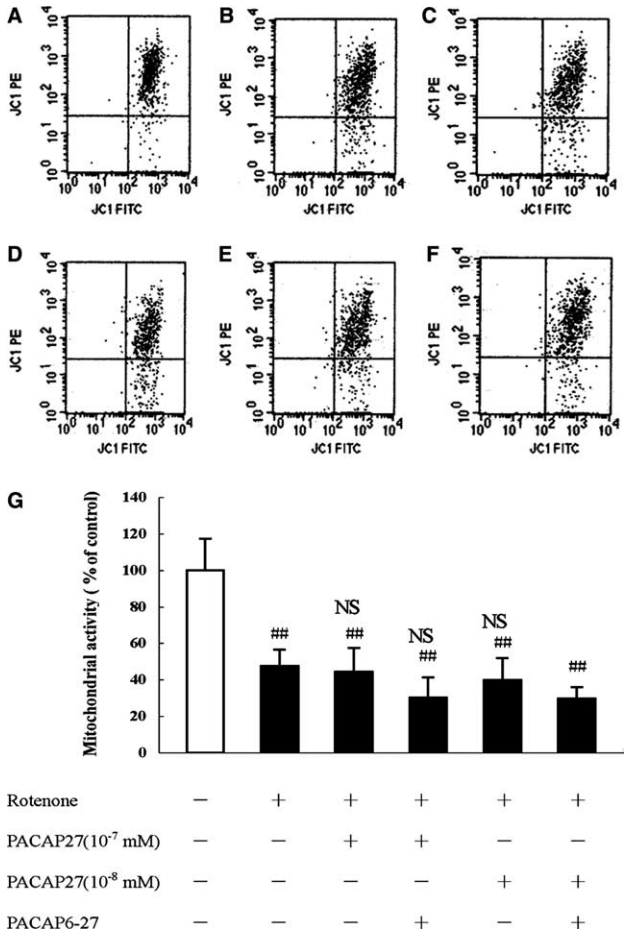


Fig. 5. PACAP27 effects on the alteration of mitochondrial membrane potential induced by rotenone. (A)–(F) Flow cytometry data with JC-1. (A) Control group. (B) Rotenone(250 nM)-treated group. (C) PACAP27 (10^{-7} M)-treated group with rotenone (250 nM). (D) PACAP27 (10^{-7} M)-treated group with rotenone (250 nM) in the presence of PACAP6-27 (10^{-5} M). (E) PACAP27 (10^{-8} M)-treated group with rotenone (250 nM). (F) PACAP27 (10^{-8} M)-treated group with rotenone (250 nM) in the presence of PACAP6-27 (10^{-5} M). (G) Mitochondrial integrity in PC12 cells. Data are presented as means ± S.D. of four experiments. $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ vs. the control group. NS, not statistically different vs. rotenone-treated group.

neuroprotective/neurotrophic factor. This neuroprotective effect was attenuated by PACAP6-27, a potent PAC1 receptor antagonist. Taken together with previous observations that show that PC12 cells express PACAP-specific receptor PAC1-R, these data indicate that the neuroprotective effect of PACAP against rotenone-induced apoptosis of PC12 cells is mediated through activation of PAC1-R.

Our results indicate that PC12 cells are sensitive to 24 h exposure to rotenone at 50 nM to 5 μM. This finding differs from previous reports [23,24] that 0.03–1 μM of rotenone for 24 h could not influence cellular viability. This discrepancy could be due to the type of PC12 cells used or to differences in the culture conditions. There are no reported differences in release, storage or uptake of catecholamines between neuronal differentiated PC12 cells and undifferentiated PC12 cells. However, nerve growth factor (NGF) has been reported to increase electrical excitability and acetylcholine sensitivity in undifferentiated rat PC12 cells [25]. The PC12 cells in this study were differentiated by NGF, whereas Tai and colleagues used undifferentiated cells. Thus, our results reveal differential susceptibility of undifferentiated versus differentiated PC12 cells to rotenone-induced neurotoxicity, with the latter more sensitive to neurotoxins.

The effect of PACAP27 was mimicked by both a cell permeant cAMP analog (db-cAMP) and a potent PKA stimulator (forskolin). However, a PKC stimulator (TPA) had no effect. Treatment with a PKA inhibitor (H89), but not a PKC inhibitor (Myr-Ψ PKC), attenuated PACAP-evoked neuroprotection. These results suggest that the neuroprotection of PACAP against rotenone-induced cell death can be accounted for by activation of the AC but not phospholipase C/PKC signaling pathway.

In addition to the PKA signaling pathway, MAPK is a key regulatory enzyme in the final common signaling pathway for PACAP27-induced cellular proliferation, differentiation and neuroprotection in PC12 cells, and this kinase is activated by PKA followed by the activation of adenylate cylase [26,27]. In the MAPK signaling network, the ERK, JNK and p38 MAPK pathways are thought to influence the fate of PC12 cells [28]. We observed that the protective effect of PACAP against rotenone-induced cell death was blocked by administration of an ERK inhibitor or a selective p38 MAPK inhibitor. However, a JNK inhibitor had no effect. Hence, the observed PACAP neuroprotection appears to be mediated via PKA signaling and to involve downstream activation of ERK and p38 signals.

Apoptosis is a physiologically important cellular suicide pathway and there is ample evidence indicating apoptosis as a mechanism of neuronal cell death in PD. Indeed, apoptotic cells have been observed while examining PD related models in vivo and in vitro and in brains of PD patients[29]. Apoptosis is mediated by the family of cysteine, aspartyl-specific proteases known as caspases, and among these caspases, caspase-3 plays a fundamental role in the apoptosis of PC12 cells. Caspases are activated following various neuronal insults [16,18,30]. In this study, rotenone increased basal caspase-3 activity up to 139% of control levels. After the addition of PACAP27, there was a significant decrease in the catalytic activity of caspase-3, suggesting that PACAP27 acted as an anti-apoptotic agent through the deactivation of caspase-3. Caspase-3 can be activated through mitochondrial-independent or -dependent pathways involving inductive caspases such as caspase-8 or caspase-9, respectively [31].

There is little research focused on the relationship between caspase-3 activity and mitochondrial membrane integrity in the neuroprotection evoked by PACAP27 in cells. Recently, one study reported that although PACAP inhibited caspase-3 activity, it did not prevent the deleterious effects of Aβ25–35 on mitochondrial potential and granule cell death [30]. Measuring mitochondria activity with the membrane potential-sensitive probe JC-1 revealed that rotenone strongly decreased the proportion of active mitochondria. This effect was not affected by PACAP. These results suggest that activation of caspase-3 by rotenone and inhibition of caspase-3 activity by PACAP27 could be mediated through a mitochondrial-independent pathway. Additionally, there may be different relationships between caspase-3 activity and mitochondrial membrane integrity in non-PC12 cells exposed to neurotoxins. The degree of involvement of caspase-3 activation in neurotoxin-induced apoptosis may be related to cell type and the specific neurotoxin employed. Thus, further studies are needed to fully characterize mechanisms underlying protection against neurotoxicity.

In conclusion, our results demonstrate for the first time that PACAP27 has a protective role in neuronal differentiated PC12 cells against rotenone-induced neurotoxicity. We further demonstrated that the neuroprotective effects of PACAP are associated with both activation of MAPK by PKA and inhibition of caspase-3 activity. The neuroprotective effects of PACAP appear to be mediated through a mitochondrial-independent pathway.

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